The absence of detectable methylated bases in *Drosophila melanogaster* DNA

Simcha Urieli-Shoval, Yosef Gruenbaum, John Sedat+ and Aharon Razin

Department of Cellular Biochemistry, The Hebrew University Medical School Jerusalem, 91010 Israel and + *Department of Biochemistry and Biophysics, School of Medicine, University of California, San Francisco, CA 94143, USA*

Received 16 June 1982; revision received 19 July 1982

DNA methylation *5-Methylcytosine* *6-Methyladenine* (Drosophila melanogaster *salivary glands)* *Nearest neighbor analysis*

1. INTRODUCTION

Most of the eukaryotic organisms are methylated at specific cytosine residues in their DNA. For more than 2 decades efforts have been made to answer the question of whether the DNA of the fruit fly *Drosophila melanogaster* is methylated, but the results were inconclusive. The answer to this question is now attracting special interest in light of the fact that in recent years substantial evidence has accumulated, suggesting a correlation between vertebrate DNA methylation and gene expression [1]. *Drosophila* in particular is an interesting organism in this respect as it goes through several defined developmental stages and its genome organization has been extensively investigated. Here, a variety of highly sensitive methods have been used to analyze methylated bases in *Drosophila melanogaster* DNA. 5-Methyl-cytosine, which is the common methylated base in DNA of eukaryotic organisms, could not be detected in any of the developmental stages of this organism. There is no indication for other modifications of this DNA as well. The well-defined clonally inherited patterns of methylation of the genetic material in mammals [1] and the absence of such methylation patterns in *Drosophila* DNA suggested here, bring into focus the long-standing enigma of the biological role played by methylation patterns.

2. MATERIALS AND METHODS

2.1. *Preparation of DNA*

DNA of *Drosophila melanogaster* embryos, larvae, pupae and adults was prepared from isolated nuclei. The nuclei were lysed in 1 mM Tris (pH 8) and after centrifugation at 10 000 × *g* the chromatin pellet was suspended in a lysis mixture containing 0.5% (w/v) sodium lauryl sulphate; 2.5 mM EDTA; 0.5 M NaCl; 10 mM Tris (pH 8) and 100 μg proteinase K/ml (Merck Co.). The mixture was incubated for 2 h at 37°C and RNase treated (300 μg pancreatic RNase/ml for 1 h at 37°C). Phenol extraction was followed by chloroform:isoamyl alcohol (24:1, v/v) extraction and ethanol precipitation. The DNA was hydrolyzed to free bases for analysis as in [2].

2.2. *Nearest neighbor analysis*

The DNA was dissolved (1 mg/ml) in 5 mM Tris (pH 8), 0.1 mM EDTA and subjected to the modified nearest-neighbor method [8]. DNA samples were nicked by pancreatic DNase I and the nicked DNA was incubated for 10 min at 15°C in a reaction mixture containing 50 mM potassium phosphate (pH 7.2). 5 mM $MgCl_2$, 1 mM mercaptoethanol, 7 units *E. coli* DNA *Pol*I and 1.2 μM d[α^{32}P]GTP (670 Ci/mmol). The unreacted d[α-^{32}P]GTP was removed by Sephadex G-50 chromatography. The labelled DNA was digested to deoxynucleoside-3'-monophosphates by micrococcal nuclease (140 μg/ml) and spleen phosphodi-

Published by Elsevier Biomedical Press

esterase (7 units/ml) for 3 h at 37°C and the products separated by two-dimensional thin-layer chromatography.

3. RESULTS AND DISCUSSION

In an effort to detect 5-methylcytosine (m^5Cyt) in *Drosophila* DNA 3 highly sensitive methods were used: high resolution mass spectrometry [2], gas chromatography–mass spectrometry [3] and high-performance liquid chromatography [4]. In none of these methods could m^5Cyt be detected (not shown). The conclusion of those results was that the content of m^5Cyt, if present in *Drosophila melanogaster* DNA, is below the limit of sensitivity of the methods used (0.1% m^5Cyt of total Cyt residues in the DNA). This unexpected observation indicated that *Drosophila melanogaster* is different from other eukaryotes with regard to the pattern of DNA methylation.

Since eukaryotic DNA in general is known to be methylated at cytosine residues which are located at CpG sequences, the sensitivity of detection of m^5Cyt would have been several fold higher were CpG sequences analyzed rather than total Cyt residues. One way to probe methylation at CpG sequences was based on the use of CpG restriction enzymes such as *Hpa*II, *Msp*I and *Hha*I [5–7]. *Hpa*II and *Msp*I recognize the sequence CCGG. *Hpa*II does not cleave when the internal cytosine residue is methylated, whereas *Msp*I is sensitive to methylation at the external Cyt. *Hha*I does not cleave the methylated GCGC sequence. Results of the experiments using CpG enzymes were all

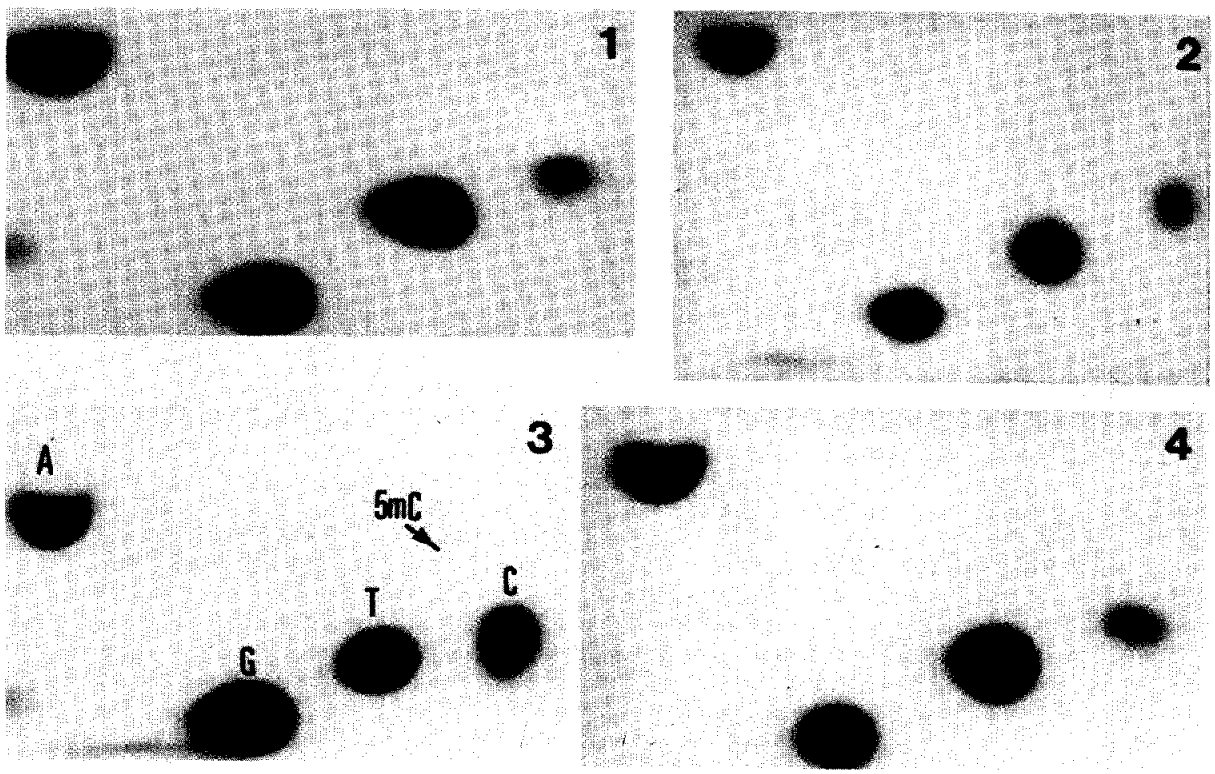

Fig.1. Analysis of m^5Cyt at CpG sequences of DNA from embryos, larvae, pupae and adult *Drosophila melanogaster*. DNA was prepared and subjected to nearest neighbor analysis, as in section 2. The autoradiographs represent analyses of DNA from: (1) embryo; (2) larvae; (3) pupae; (4) adult. The radioactive spots were scraped off and counted by liquid scintillation. The counts found in dCMP were 7148, 31 738, 27 681 and 7888 for embryo, larvae, pupae and adult DNA, respectively. Background counts, at non-radioactive areas on the plate were ≤ 10 cpm (100 cpm/10 min above counter background). *Abbreviations:* A, dAMP; G, dGMP; T, dCMP; 5mC, 5mdCMP.

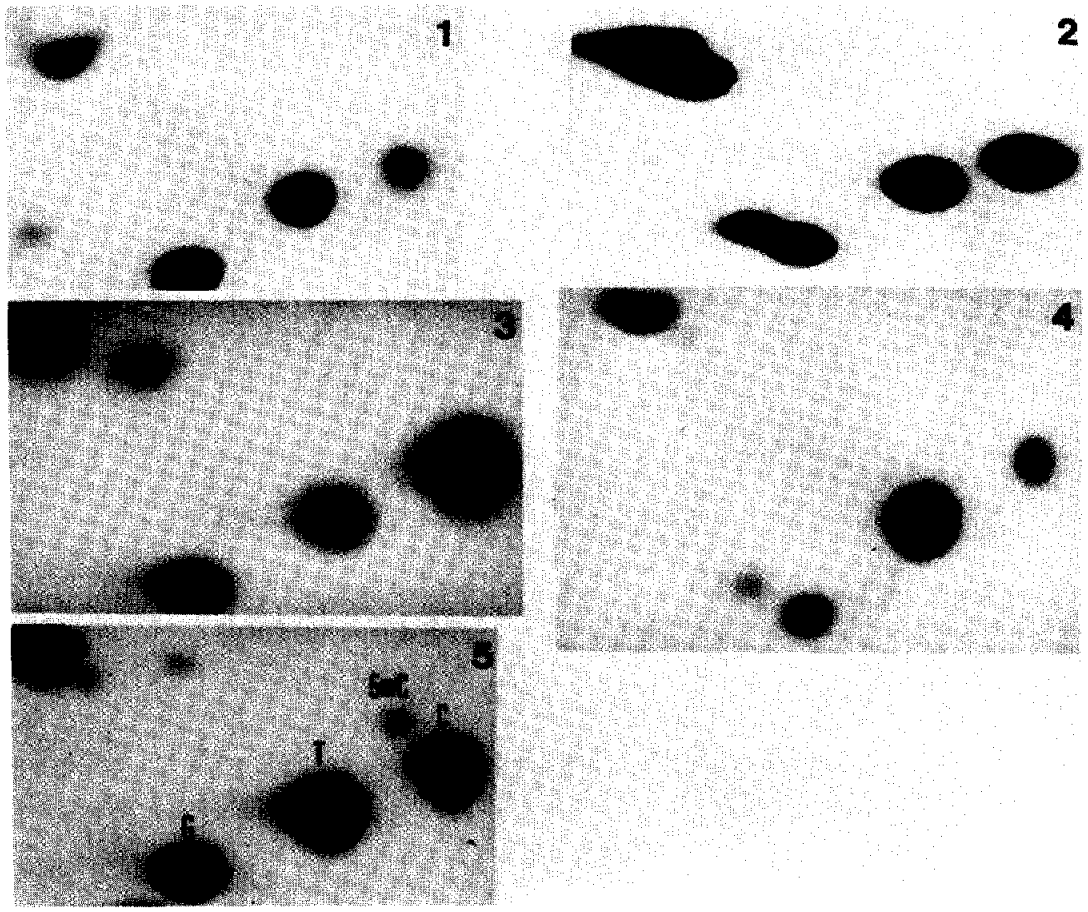

Fig.2. Analysis of m^5Cyt at CpG, CpC, CpA and CpT sequences of DNA from salivary glands. Third instar larvae were used to dissect 800 salivary glands which were collected in buffer containing 0.1% digitonin and 15 mM Pipes buffer (pH 7.2) and stored at −70°C until used. Lysis mixture was added and the DNA preparation proceeded as in section 2. DNA was subjected to nearest neighbor analysis based on nick translation with a single d[α-^{32}P]NTP at a time described in section 2. Autoradiograms represent: (1) labelling with d[α-^{32}P]GTP; (2) labelling with d[α-^{32}P]ATP; (3) labelling with d[α-^{32}P]CTP; (4) labelling with d[α-^{32}P]TTP; (5) *E. coli* C DNA, labelling with d[α-^{32}P]TTP. The radioactive spots were scraped off and counted by liquid scintillation. The counts found in dCMP were: 95 000, 14 000, 5618, 2916 and 2500 for 1–5, respectively, whereas 96 counts were found in the m^5Cyt spot of *E. coli* DNA. *E. coli* C DNA contains methyl groups at the internal cytosine residue of CC$\overset{\text{A}}{\underset{\text{T}}{}}$GG sequences. Nearest neighbor analysis with d[α-^{32}P]TTP reveals 50% of the *E. coli* DNA m^5Cyt residues, comprising 4% of the CpT sequences (in preparation). In addition to the 5 nucleoside-3′-monophosphates we frequently obtained several additional spots, including one that migrates close to adenosine-3′-monophosphate which was identified as adenosine-5′-monophosphate. These spots did not seem to interfere with the analysis of m^5Cyt.

negative, indicating that the sequences recognized by these enzymes are not methylated in *Drosophila* DNA. However, this method probes only for a subset of the CpG sequences. The analysis of m^5Cyt in CpG sequences as a whole has been achieved using the nearest neighbor technique that we have developed [8]. By this method we could show that CpG sequences are not methylated in *Drosophila melanogaster* embryos, pupae, larvae and adult DNA ($\leq$0.1% of the CpG sequences or $\leq$ 1 m^5Cyt residue/10 kb) (fig.1). Similar analyses of DNA from the *Drosophila* KC cell line and 90,

170 min and 7 h embryos also revealed no m^5Cyt.

A recent publication claiming *Diptera* salivary gland polytene chromosome DNA to be methylated, suggests an association of methylation with polytenization [9]. This claim was based on the analysis of polytene chromosomes for their content of m^5Cyt by anti-m^5Cyt antibodies. However, in the same study using CpG restriction enzymes, the investigators detected no methylation in polytene DNA at CpG sequences. Although the m^5Cyt residues in DNA of eukaryotic organisms are almost exclusively present in CpG sequences [1], we have found that m^5Cyt residues in higher plant DNA can also be found in CXG sequences, where X can be A, T or C [10]. Therefore, it was necessary to examine the possibility of the presence of m^5Cyt at CpA, CpT or CpC sequences in the polytene DNA of the salivary glands. We have analyzed salivary gland DNA for the presence of m^5Cyt in CpG, CpC, CpA and CpT sequences using the nearest neighbor method [8]. The results reveal that < 1 Cyt residue/10 kb is methylated in salivary gland DNA (fig.2). The same results were obtained with total DNA from embryo, pupae, larvae and adults.

Taking all the results together, it can be concluded that in contrast to most eukaryotic organisms, *Drosophila melanogaster* and perhaps other insects are devoid of m^5Cyt in their DNA. However, we cannot rule out 1 methylated base/band. The fact that *Drosophila* DNA is undermethylated compared to mammalian DNA raises the question of the mechanism that could substitute in this organism for the role played by the DNA methylation pattern that is known now to be tissue specific and clonally inherited in mammalian DNA. In mammals, the methylation pattern is clonally inherited and that methylated cytosine residues in gene sequences may block the expression of the gene [11]. It was still possible that other methylated bases replace m^5Cyt in *Drosophila*. Base composition analysis of *Drosophila* DNA of all developmental stages by HPLC revealed no other minor base (not shown). However, since a study on DNA from mosquito cell line [12], and another study on *Tetrahymena pyriformis* DNA [13] report the presence of 6-methyladenine (m^6Ade) in these DNAs it seemed necessary to examine the possibility of the presence of low levels of m^6Ade in *Drosophila* DNA. We have analyzed adult *Drosophila* DNA for the presence of m^6Ade by the modified nearest neighbor analysis [8] and found no indication for the presence of m^6Ade in ApA, ApC, ApG or ApT sequences (< 1 m^6Ade residue/10 kb).

Based on recent data in eukaryotes, it is believed that the methylation pattern is laid down by specific demethylation early in development at a predetermined stage [1]. The established differentiated methylation pattern is then stably inherited in the somatic cell lines for many generations [14] by a maintenance methylase [15]. The absence of DNA methylation in *Drosophila* suggests that whatever the clonal inherited pattern of DNA methylation in mammalian DNA reflects, it must be substituted by some other, yet unknown, mechanism in *Drosophila*. This hypothetical mechanism might be based on the same, as yet undiscovered, molecular basis for determining the differentiated methylation pattern of DNA in mammals.

ACKNOWLEDGEMENTS

This work was supported by NIH grant GM 20483. We are grateful to Dr Judy Singer for help with the GC–MS determinations, to Dr Igor Dawid for invaluable criticism and *Drosophila* DNA samples during the early stages of this study and to Dr H. Cedar for helpful discussions.

REFERENCES

[1] Razin, A. and Riggs, A.D. (1980) Science 210, 604–610.
[2] Deutsch, J., Razin, A. and Sedat, J.W. (1976) Anal. Biochem. 72, 586–592.
[3] Singer, J., Schnute, W.C. jr, Shively, J.E., Todd, C.W. and Riggs, A.D. (1979) Anal. Biochem. 94, 297–301.
[4] Singer, J., Stellwagen, R.H., Roberts-Ems, J. and Riggs, A.D. (1977) J. Biol. Chem. 252, 5509–5513.
[5] Cedar, H., Solage, A., Glaser, G. and Razin, A. (1979) Nucleic Acids Res. 6, 2125–2132.
[6] Bird, A.P. and Taggart, M.H. (1980) Nucleic Acids Res. 8, 1485–1497.
[7] Rae, P.M.M. and Steele, R.E. (1979) Nucleic Acids Res. 6, 2987–2995.
[8] Gruenbaum, Y., Stein, R., Cedar, H. and Razin, A. (1981) FEBS Lett. 124, 67–71.
[9] Eastman, E.M., Goodman, R.M., Erlanger, B.F. and Miller, O.J. (1980) Chromosoma 79, 225–239.
[10] Gruenbaum, Y., Naveh-Many, T., Cedar, H. and Razin, A. (1981) Nature 292, 860–862.

[11] Stein, R., Razin, A. and Cedar, H. (1982) Proc. Natl. Acad. Sci. USA 79, 3418–3422.
[12] Adams, R.L.P., McKay, E.L., Craig, L.M. and Burdon, R.H. (1979) Biochim. Biophys. Acta 563, 72 81.
[13] Hattman, S., Kenny, C., Berger, L. and Pratt, K. (1978) J. Bacteriol. 135, 1156–1157.
[14] Stein, R., Gruenbaum, Y., Pollack, Y., Razin, A. and Cedar, H. (1982) Proc. Natl. Acad. Sci. USA 79, 61–65.
[15] Gruenbaum, Y., Cedar, H. and Razin, A. (1982) Nature 95, 620 622.